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Independently gated multiple substates of an epithelial chloride-channel protein

(reconstitution/ion channels/monoclonal antibodies)

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ABSTRACT **We have purified a protein from *Necturus maculosus* gallbladder cells that forms chloride channels in an artificial membrane. The same protein apparently can form channels that are highly selective for chloride but can have conductances varying from 9 to about 150 pS. The high-conductance channels are blocked by the monoclonal antibody used to purify the protein, but this antibody has no effect on the 9-pS channels. The observation that gating of the low- and high-conductance states is independent and that the antibody affects only the latter has implications regarding the control of chloride conductance in cell membranes and the different types of channels described in those cells.**

Chloride channels have been studied in a wide variety of cells (1–8) and have been reconstituted in artificial membranes (9–11); cDNA encoding chloride channels have been cloned and expressed (12, 13). In addition, cloning of the cDNA encoding cystic fibrosis transmembrane regulatory protein (14–16) has led to the discovery that this protein is probably also a chloride channel (17–19). Despite this body of work, many questions remain about the functional properties of epithelial chloride channels. We have previously reported the partial purification and reconstitution of a chloride-channel protein from *Necturus maculosus* gallbladder (20), which, although highly selective for chloride and partially blocked by the antibody in a lipid bilayer, demonstrated multiple, almost chaotic, current levels. We now show that no matter the stage of purification, this channel can adopt different conductances, the smallest of which is 9 pS, and that channels of this size are regulated differently from the more conductive ones in that only the latter are blocked by antibody.

METHODS

Protein Purification. Cells from ≈25 *N. maculosus* gallbladders were scraped from untreated tissues into 7 ml of phosphate-buffered saline (PBS) containing 10 mM Na_3PO_4, 150 mM NaCl, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 0.1 mM leupeptin, and 1 mM pepstatin. Cells were disrupted by nitrogen cavitation, and membrane proteins were solubilized in 10 mM 3-[3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS). Cellular debris was pelleted at 10,000 rpm for 20 min at 4°C. The supernatant was filtered through a 0.2-μm syringe filter, and 25–30 mg of protein was loaded onto a 20-ml Sephacryl S-200 column in 0.1% triethanolamine/1% CHAPS for desalting and partial size selection. Approximately 20 mg was contained in the 8-ml high molecular weight fraction [which contains the epitope, as confirmed by immunoblot (Western) analysis]. This fraction was separated by fast protein liquid chromatography (FPLC) with a Mono Q column (Pharmacia) in 0.3% triethylamine/1% CHAPS with a linear NaCl gradient of 0–1.2 M. The high molecular weight immunoreactive band was eluted at ≈0.6 M NaCl. However, this peak still contained a number of protein bands. The final step of purification was immunoaffinity column chromatography. The column was prepared by purifying IgG with a Bio-Rad Affi-Gel protein A Maps II kit; the purified IgG was then coupled to Affi-Gel Hz hydroxide gel with Bio-Rad's Affi-Gel Hz immunoaffinity kit. The FPLC Mono Q peak fraction (4 ml) was diluted to 10 ml, Na_3PO_4 was added to 50 mM, and protein was bound to the immunoaffinity column overnight at 4°C. The column was washed with 40 ml of PBS containing 0.5 M NaCl and 1% CHAPS, and the immunoreactive protein was eluted with 15 ml of 3 M KSCN/0.5 M imidazole/0.5 M NaCl/0.1% Triton X-100. Silver staining was done with a Bio-Rad Silver Stain kit, and Western blotting was done as described (20).

Reconstitution and Recording. Planar lipid bilayers were formed from a mixture of palmitoyl/oleoyl phosphatidylethanolamine and palmitoyl/oleoyl phosphatidylserine as described (21). We added solubilized protein (5–15 μl) directly to the cis solution as previously described; after incorporation and recording, the concentration of sodium chloride in that chamber was changed to a final value of 150 or 200 mM. Antibody (as obtained from mouse ascites fluid and stored frozen) was added to the trans side (it has no effect from the cis side) at a 1:100 dilution.

Bilayers were clamped at voltages from −100 to +100 mV, with the voltage defined as cis–trans (ground). At positive holding potentials, currents flowing from cis to trans represent "outward" currents and are shown as upward transitions in the recordings. Currents were recorded at a bandwidth of 2000–3000 Hz and stored on a video cassette recorder; subsequently they were filtered at 200 Hz, digitized at 800 Hz, and analyzed.

RESULTS AND DISCUSSION

We prepared a monoclonal antibody that recognizes the cell membranes from the gallbladder of *N. maculosus* (21) and used the antibody to prepare an immunoaffinity column. Using solubilized protein from the gallbladder cells, we have been able to purify the protein to apparent homogeneity (Fig. 1).

In an earlier study we reconstituted partially purified protein into planar lipid bilayers and showed that this material forms a highly chloride-selective channel that is at least partly blocked by the monoclonal antibody used to isolate the protein (20). Although ion selectivity could be measured, kinetics of the channel were impossible to obtain because of the very complicated behavior it displayed. Furthermore, owing to the appearance of such large and complicated channels, we were unable to observe smaller channels, since

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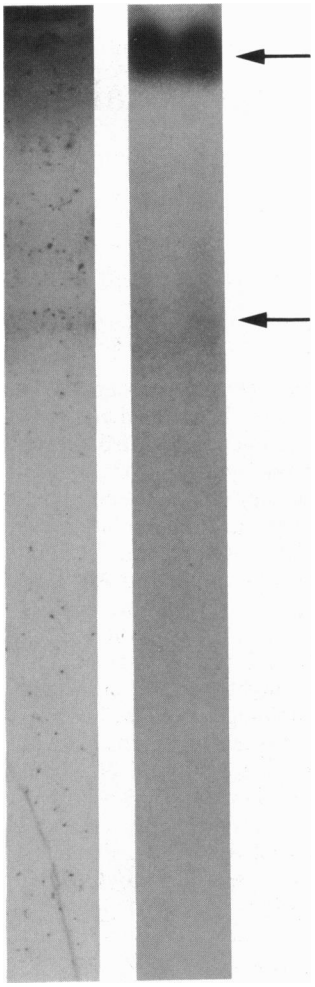

FIG. 1. Silver-stained gel (*Left*) and Western blot (*Right*) that show the same bands. The major band (upper arrow) is the protein (≈219 kDa) previously described. The light band (lower arrow) at about 85 kDa appeared only during late stages of purification, and is probably a degradation product; as shown, it is also immunoreactive.

recording at high gain was impossible. One possible cause of this complex behavior was that different molecular species were being incorporated. However, our experiments indicate that the results were identical regardless of the stage of purification. It is not likely that the smaller band seen in Fig. 1 is the channel-forming moiety, since we never see a band of this size on Western blots during earlier stages of purification when, as noted above, the reconstituted channel is indistinguishable from that seen with the purified preparation. That is, no differences can be detected whether we use (*i*) material eluted after one-step purification from the immunoaffinity column (Figs. 2*B* and 3), (*ii*) that obtained from the center of the only peak seen on FPLC purification of the immunoaffinity eluate (20), or (*iii*) the apparently homogeneous material (Fig. 1) used for most of the experiments presented in this paper (Figs. 2 *A* and *C* and 3–6).

We found that we could resolve smaller channels by perfusing the chamber at an early time after insertion: we repeatedly observed insertion of a far smaller channel generally before the larger ones appeared (Fig. 2*A*). Once it became clear that small channels were in fact present, experiments could be done without perfusion, and recordings were made at high gain from the start. In some experiments we saw multiple small channels without the appearance of the larger ones (Fig. 2*B*), and in others (Fig. 2*C*) we saw both small and large channels (or substates) open at the same time. The mean conductance of the small channel is 9 pS, and the reversal potential indicates that it is highly selective for chloride (Fig. 3) as had been the case for the channel previously reported. Unlike the case for the high-conductance-state channels (see below and ref. 20), the antibody has no effect on the single channels; furthermore, there is no effect of 4,4′-dinitrostilbene-2,2′-disulfonic acid or 5-nitro-2-(3-phenylpropylamino)benzoic acid at concentrations up to 200 μM—chemicals which have been shown to block some other chloride channels at these concentrations, on either the high- or the low-conductance channels.

The basic unit appears to have a conductance of 9 pS, yet the apparently pure protein also routinely shows multiple states. Several possibilities exist: (*i*) two different protein species insert into the bilayer, (*ii*) several identical molecules (or subunits of that molecule) insert, or (*iii*) a single molecule displays multiple substates. The first possibility seems unlikely because both the small and large channels show 20-fold selectivity for chloride over sodium and because once we started seeking the small channel, we have almost never seen large-channel insertions without one or more 9-pS units also evident. As to the second possibility, if we are to assume that all of these states represent aggregates of the 9-pS unit, then there must be, in the bilayers, up to 30 such units. Since the conductance in any bilayer does not usually increase progressively with time (that is, the maximum conductance occurs within at most a few minutes after the first channel is seen and then remains stable) and since the bilayer is vast in size compared with a single molecule, these observations in addition to the apparent cooperative gating imply that any such aggregation would occur before the protein enters the bilayer. Finally, there is both precedent (22) and theory (23) consistent with the appearance of multiple substates of a single molecule, presumably because of small changes in conformational states of the channel. The results of studies with monoclonal antibody E12, the antibody used to purify the protein, are most consistent with the last possibility.

This antibody blocks the chloride conductance in a number of tissues from mudpuppy to man (20, 21) and also markedly reduces the number of open states present in the bilayer (Figs. 4 and 5). However, the antibody has no effect on the 9-pS channels; indeed, addition of antibody (and it is active only from the trans side) in the presence of large channels leads to enough of a decrease in conductance levels that it is again possible to see 9-pS channels (Fig. 6), although multiple-level gating is not fully abolished.

Multiple substates have been observed many times, both from patch-clamp recording of either cation (24, 25) or anion (26) channels and from reconstitution experiments using either impure (27–29; reviewed in ref. 30) or apparently pure (22) membrane proteins incorporated into lipid bilayers. Although in some instances the multiple substates show similar ion selectivities, different regulatory mechanisms accounting for the gating of such substates have not been demonstrated.

The mechanism of such gating is not clear, although it has been shown (23) on theoretical bases that a number of subconductance states can exist in a single channel that assumes small changes in conformation or electrostatic properties. Were the channel to aggregate in the bilayer, antibody might bind in some way and prevent the aggregation or the opening of a "main" gate. Such a gate would have its own kinetics and upon opening would yield total conductances that depend on the number of individual units open at the time. Such a mechanism could explain the frequency of channel transitions that span several units at one time, as shown for the chloride channel discussed here. However, as noted above, aggregation within the bilayer is unlikely. A more likely mechanism is that antibody binds to a region of

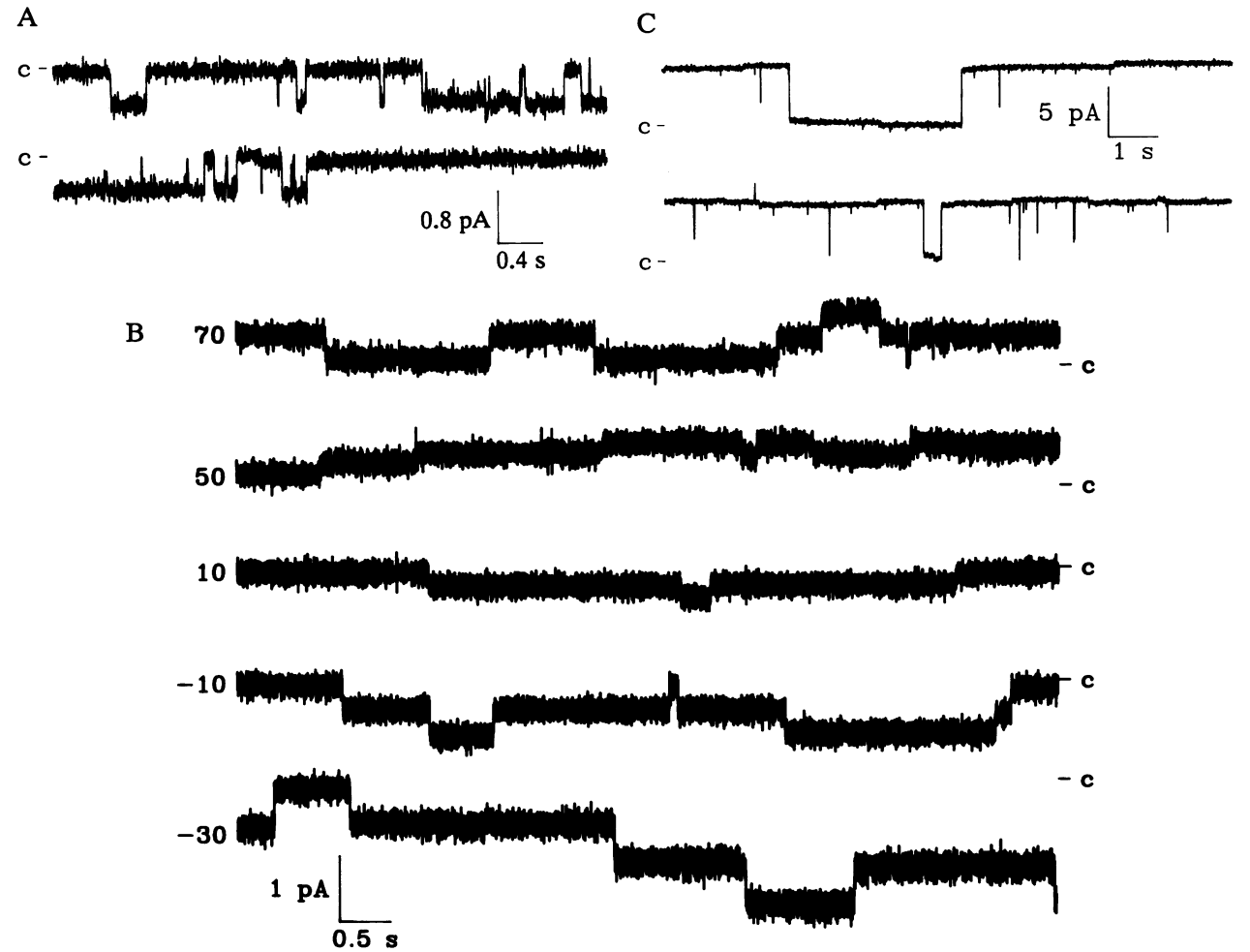

FIG. 2. (*A*) Trace of a single channel in a bilayer. (*B*) Recordings at several voltages of an experiment in which at least three small channels are present. (*C*) Trace showing a single 10-pS channel superimposed on one of about 150 pS. The larger transitions appeared several minutes after the smaller ones. Addition of antibody had no effect on these channels. c, Closed state.

the channel molecule to prevent or limit the changes in conformation required to form the large conductance states.

It is worthwhile to consider the possible relevance of the antibody effects to the physiological state of the native channels. Although monoclonal antibody blocks the chloride conductance in a number of tissues, we always used agonist (theophylline in the case of *N. maculosus* gallbladder) in submaximal doses; however, blockade can always be overcome by a supramaximal dose of agonist. The present ex-

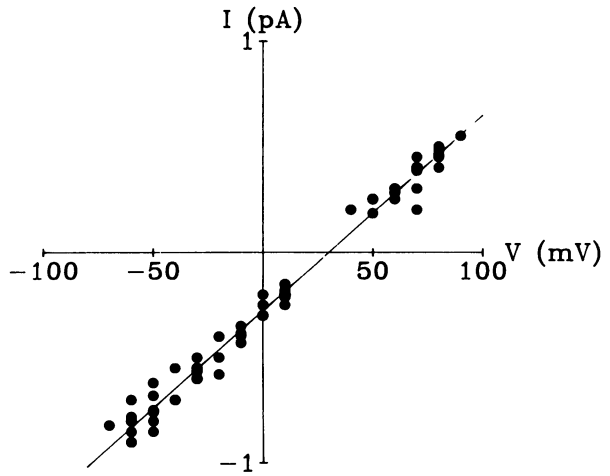

FIG. 3. I–V curve constructed from 10 experiments like those shown in Fig. 2*B*. The slope conductance is 9.1 ± 0.3 pS, and the reversal potential with a 200–50 mM NaCl gradient is 30.8 mV, indicating a P_{Cl}/P_{Na} ratio of 19.4, where P is permeability.

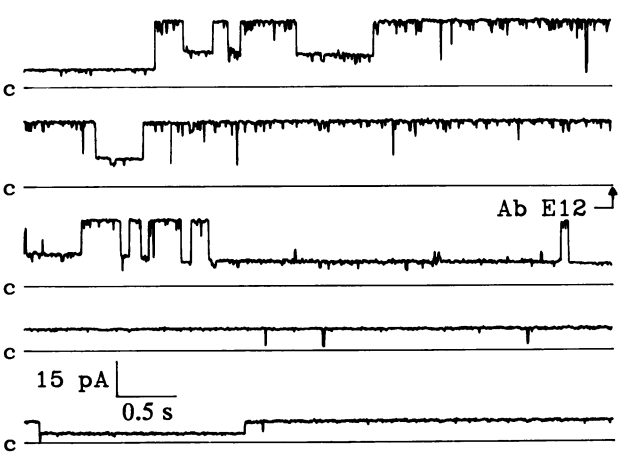

FIG. 4. Continuous tracings of multiple large channels before and after antibody addition. The lines represent the zero current level and are included to clarify the point that we never see complete closing of all channels when multiple channel gating is present. Antibody is added at the end of the second trace, and the mixture is stirred for 30 s (record not shown). There clearly is an effect, which begins soon after the start of the third trace; by the end of the last trace, most of the large transitions have disappeared.

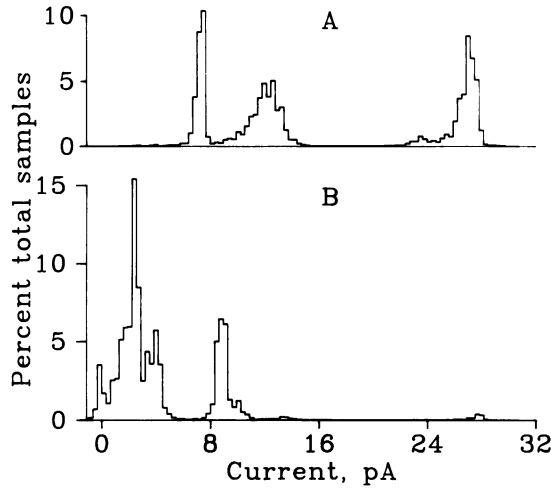

FIG. 5. Amplitude histogram of all data at this potential (60 mV), both before (*A*) and after (*B*) the addition of antibody, indicating a marked shift to smaller transitions and some complete closures.

periments suggest a possible explanation for that finding. If the channel can exist in both low- and high-conductance conformations (so they appear to gate either individually or as aggregates) and if antibody blocks the high-conductance conformation, one reasonable explanation for the findings in the cells is that increasing amounts of agonist cause insertion or activation (or both) of more and more channels. If enough low-conductance channels form and remain as such, it could lead to an increase in membrane conductance that is antibody-independent, since antibody affects only the ability to form the high-conductance states. Such a phenomenon could also lead to different control mechanisms for total-cell chloride conductance, since the high- and low-conductance channels could be controlled separately. Of interest in this regard is that we have shown recently (31) that the high-conductance channel is regulated in the bilayer by phosphorylation and dephosphorylation. However, there is no effect of cyclic AMP-dependent protein kinase on the 9-pS channel, again indicating independent regulation of the gating mechanisms for the single channel and for the aggregates. This type of mechanism may also explain the multiple conductance states seen in other cell membrane channels and may indicate that

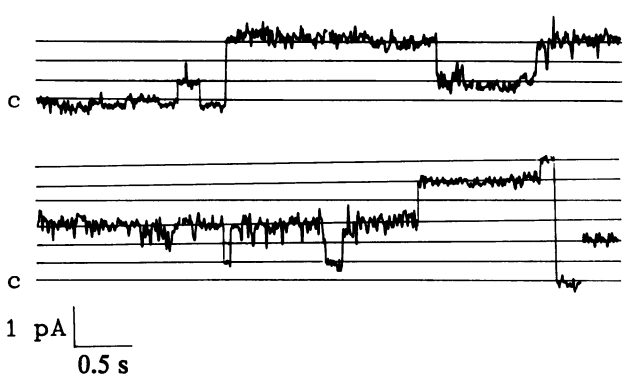

FIG. 6. Traces 5 min after antibody addition at a potential of −50 mV. This record is highly filtered because of the low gain used in the experiment. The lines are drawn to be equidistant between the zero current value and the largest current seen in the bilayer, indicating the presence of six channels, each with a conductance of 8.6 pS. Although there are still several transitions that include multiple units, there are no current levels any higher than these.

separate control mechanisms exist for single channels and for aggregates or other cooperative conductance states of those channels.

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1. Goegelein, H. (1988) *Biochim. Biophys. Acta* **947**, 521–547.
2. Duszyk, M., French, A. S. & Man, S. F. P. (1990) *Biophys. J.* **57**, 223–230.
3. Marunaka, Y. & Eaton, D. C. (1990) *Am. J. Physiol.* **258**, C352–C368.
4. Geletyuk, V. I. & Kazachenko, V. N. (1985) *J. Membr. Biol.* **86**, 9–15.
5. Miller, C. (1982) *Philos. Trans. R. Soc. London B.* **299**, 401–412.
6. Tabcharani, J. A., Low, W., Elie, D. & Hanrahan, J. W. (1990) *FEBS Lett.* **270**, 157–164.
7. Duszyk, M., French, A. S. & Man, S. F. P. (1992) *Biophys. J.* **61**, 583–587.
8. Gray, M. A., Pollard, C. E., Harris, A., Coleman, L., Greenwell, J. R. & Argent, B. E. (1990) *Am. J. Physiol.* **259**, C752–C761.
9. Dubinsky, W. P. & Monti, L. B. (1986) *Am. J. Physiol.* **251**, C713–C720.
10. Valdivia, H. H., Schultz, S., Dubinsky, W. P. & Coronado, R. (1988) *Pediatr. Pulmonol. Suppl.* **2**, 103 (abstr.).
11. Goldberg, A. F. X. & Miller, C. (1991) *J. Membr. Biol.* **124**, 199–206.
12. Steinmeyer, K., Ortland, C. & Jentsch, T. J. (1991) *Nature (London)* **354**, 301–304.
13. Jentsch, T. J., Steinmeyer, K. & Schwarz, G. (1990) *Nature (London)* **348**, 510–514.
14. Rommens, J. M., Iannuzzi, M. C., Kerem, B., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J. R., Tsui, L.-C. & Collins, F. S. (1989) *Science* **244**, 1059–1065.
15. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M., Collins, F. S. & Tsui, L.-C. (1989) *Science* **244**, 1066–1073.
16. Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M. & Tsui, L.-C. (1989) *Science* **244**, 1073–1080.
17. Kartner, N., Hanrahan, J. W., Jensen, T. J., Naismith, A. L., Sun, S., Ackerley, C. A., Reyes, E. F., Tsui, L.-C., Rommens, J. M., Bear, C. E. & Riordan, J. R. (1991) *Cell* **64**, 681–691.
18. Arispe, N., Rojas, E., Hartman, J., Sorscher, E. J. & Pollard, H. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1539–1543.
19. Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A. E. & Welsh, M. J. (1991) *Science* **253**, 202–205.
20. Tsai, L.-M., Dillard, M., Rosenberg, R. L., Falk, R. J., Gaido, M. L. & Finn, A. L. (1991) *J. Gen. Physiol.* **98**, 723–750.
21. Finn, A. L., Tsai, L.-M. & Falk, R. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7649–7652.
22. Liu, Q. Y., Lai, F. A., Rousseau, E., Jones, R. V. & Meissner, G. (1989) *Biophys. J.* **55**, 415–424.
23. Dani, J. A. & Fox, J. A. (1991) *J. Theor. Biol.* **153**, 401–423.
24. Hunter, M. & Giebisch, G. (1987) *Nature (London)* **327**, 522–524.
25. Meves, H. & Nagy, K. (1989) *Biochim. Biophys. Acta* **988**, 99–105.
26. Krouse, M. E., Schneider, G. T. & Gage, P. W. (1986) *Nature (London)* **319**, 58–60.
27. Castillo, C., Villegas, R. & Recio Pinto, E. (1992) *J. Gen. Physiol.* **99**, 897–930.
28. Rousseau, E. (1989) *J. Membr. Biol.* **110**, 39–47.
29. Coyne, M. D., Dagan, D. & Levitan, I. B. (1987) *J. Membr. Biol.* **97**, 205–213.
30. Fox, J. A. (1987) *J. Membr. Biol.* **97**, 1–8.
31. Finn, A. L., Gaido, M. L., Dillard, M. & Brautigan, D. L. (1992) *Am. J. Physiol.* **263**, C172–C175.